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Full Length Research Paper

Over-expression of *ZmPti1*, a homologue to *Pti1*, increases salt tolerance of *Arabidopsis thaliana*

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Studies have shown that Pti1 plays an important role in plant disease resistance pathway. However, Pti1 have not been studied for its roles under salt stress condition. Previous study has shown that maize *ZmPti1* is induced by salicylic acid (SA), low-temperature, mannitol and salt. In order to analyze the further biological functions of *ZmPti1*, *ZmPti1* was over-expressed in *Arabidopsis*. Under salt stress, compared to wild type, transgenic plants grew better, had higher seedling fresh and dry weight (FW, DW), seed yields and the superoxide dismutase (SOD) activity; Seedling FW and DW of S1-1, S2-1 transgenic plants increased significantly than that of WT; Seed weight of S1-1, S2-1 transgenic plants increased 71 and 106% respectively; However malondialdehyde (MDA) content and relative electric conductivity (ion leakage) of transgenic plants were kept to a relative lower level. Based on the present knowledge, this is the first report that showed that the over-expression of *Pti1*-like gene enhances the salt resistance in plants.

Key words: ZmPti1, transgenic *Arabidopsis*, salt resistance.

INTRODUCTION

Quite a part of our world's arable land and almost a half of the irrigated agricultural land is affected by high soil salinity (Zhu, 2001). The high concentration of salt in the soil could have severe effects on plant growth and development. High salt may lead to ionic, osmotic, and oxidative stress (Zhu, 2002). High salt could also lower the osmotic potential and therefore a restricted uptake of water occurs. In addition, salt stress can cause oxidative stress because of the induction of generating reactive oxygen species (ROS) (Leshem et al., 2007). Under stress conditions, cascades or network events are activated in plants initiating with stress perception and ending with the expression of many effector genes (Pastori and Foyer, 2002). In these events, reversible protein phosphorylation catalyzed by protein kinases and protein phosphatases plays a central role (Yang et al., 1997).

The salt overly sensitive (SOS) is a well known pathway for tolerance of salt stress (Zhu, 2002; Zhu et al., 1998). In this pathway, SOS2, a member of plant SnRK3, can interact with SOS3 and then be activated. The activated SOS2 is able to phosphorylate SOS1, enhancing its activity during salt stress (Qiu et al., 2002). Moreover, recent study shows that regulation of V-ATPase activity is an additional key function of SOS2 in coordinating changes in ion transport during salt stress and in promoting salt tolerance (Batelli et al., 2007). CDPKs which also belong to CDPK-SnRK superfamily have been proven to be involved in regulating plant responses to salt stress, as well. Salinity can induce specific changes in the expression of CDPK genes in *Arabidoposis* and rice (Urao et al., 1994; Wan et al., 2007). Over-expression of OsCDPK7 could improve transgenic plants' tolerance to cold, salt and drought stresses (Saijo et al., 2000). Mitogen-activated protein kinases (MAPKs) are one of the largest family of serine/threonine kinases in higher

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Abbreviations: SA, Salicylic acid; **FW,** fresh weight; **DW,** dry weight; **SOD,** superoxide dismutase; **MDA,** malondialdehyde; **ROS,** reactive oxygen species; **SOS,** salt overly sensitive; **MAPKs,** mitogen-activated protein kinases; **MAPKK,** mitogen-activated protein kinases kinase; **MAPKKK,** mitogen-activated protein kinases kinase kinase; **HR,** hypersensitive response; **PCR,** polymerase chain reaction; **WT,** wild type; ***Pti1*,** Pto-interacting 1.

plants which play an important role in the transduction of salt stress signal. These generally function as a cascade where MAPK is phosphorylated and activated by MAPK kinase (MAPKK), which itself is activated by MAPKK kinase (MAPKKK) (Tuteja, 2007). So far, many MAP kinases in various plants including *Arabidopsis*, alfalfa, tobacco and *chorispora bungeana* have been reported to be involved in salt stress (Sanan-Mishra et al., 2006; Zhang et al., 2006a; Zhang et al., 2006b).

Pti1 (Pto-interacting 1), encoding a protein kinase, is a member of plant defense signal pathway. In this signal pathway, Pto, as a member of R gene product, can interact with the bacterial *avrPto* gene product AvrPto. This interaction of Pto-AvrPto is thought to activate the Pto kinase and induce its phosphorylation of downstream components in signaling pathways leading to defense responses (Tang et al., 1996; Scofield et al., 1996). Pto-interacting (Pti) proteins have been proven to be downstream components of Pto by using the yeast two-hybrid system. These Pti proteins included Pti1, a serine/threonine protein kinase that is specifically phosphorylated *in vitro* by Pto and is involved in the hypersensitive response (HR) (Zhou et al., 1995). Moreover, over-expression of *Pti1* in tobacco resulted in an accelerated HR when the plants were challenged with the tobacco pathogen *P. syringae* expressinging *avrPto* (Zhou et al., 1995). To date, there is no report about Pto/Pti genes involved in salt resistance. In a previous study conducted, a Pti-like kinase gene was cloned from maize named *ZmPti1*. ZmPti1 has a kinase activity *in vitro* and can be induced by salicylic acid (SA), low-temperature, mannitol and salt (Zou et al., 2006). These results indicate that *ZmPti1* may be responsible for biotic and abiotic stresses. The main goal of this study is to confirm the biological function of *ZmPti1*. Here, we demonstrate that over-expression of *ZmPti1* in *Arabidopsis* leads to enhanced salt resistance, but has no obvious effect on disease resistance.

MATERIALS AND METHODS

Plant material, transformation and growth condition

The segment *ZmPti1-dHA-His6* in expression vector p426GAL1 was amplified by PCR with adding *Bam*H I and *Pst* I sites into 5' and 3' primers, respectively, and was confirmed by sequencing. The PCR product was then digested with *Bam*H I / *Pst* I. The expression vector pGreen0029 containing a fragment *35S-C4DDPK-CBF3-NOS* was digested with *Bam*H I and *Pst* I to release the *CBF3*. The digested products were ligated with T4 DNA ligase (TaKaRa, Dalian, China). Then the recombinant vector pGreen0029 containing the fragment *35S-C4DDPK-ZmPti1-dHA-His6-NOS* was successfully constructed. The resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101 which was then used to transform *Arabidopsis* (ecotype Columbia) using the floral dip method (Clough and Bent, 1998). T_0 seeds were screened by 50 mg L^{-1} kanamycin medium. Resistant seedlings were transferred to soil in the plastic pots and grown for seeds. The T_1 seeds were subsequently selected until T_3 homozygous seeds were obtained.

The plates and pot-grown transgenic *Arabidopsis* plants were placed in growth chambers with the condition of 20℃ temperature, 100 μmol·m^{-2}·s^{-1} light, 16/8 h day/night cycle and 60% relative humidity. *Arabidopsis* wild type (WT) and T3 homozygous transgenic plants were germinated and grown in a same plastic pots containing nutrimental soil. At the four true leaves stage, the plants were thinned to one WT and one transgenic plant with same size per pot. At the six true leaves stage, disease treatment and salt treatment were started.

Western blot

About 300 mg leaves from wild-type and transgenic plants were ground in liquid nitrogen and homogenized in extraction buffer containing 10 mmol·L^{-1} Tris-HCl, pH 8.0, 0.02% NaN_3, 0.5 mM phenylmethylsulfony fluoride. After centrifugation at 16,000 g for 10 min, aliquots of supernatant were stored at -80℃. Western blot was conducted according to the protocol of Zou et al. (2006).

NaCl treatment

One-week-old WT and transgenic homozygous plants grown on vertical agar plates containing 4.4 g/L MS powder medium (Murashige and Skoog basal medium with gamborg's vitamins, Sigma) and 30 g/L surcose were transferred to the same MS agar plates supplemented with 200 mM NaCl. One week later, plants were photographed. Salt stress experiment was also conduced in growth chambers where plants were sown in 7-cm plastic pots. At the six true leaves stage, salt treatment was started by watering with 300 mM NaCl until the soil was saturated, the treatment was conducted once every three days. After two weeks of salt treatment, the plants were photographed, some plants were harvested for seedling fresh and dry weight. The fresh weight of whole plants was measured immediately after the harvest. Dry weight of whole plants was measured after 48 h at 80℃. When all the treated plants maturated, the seeds of WT and transgenic plants were harvested and weighed. Three replicates (100 plants per replicate) per line were used. Statistical differences were determined using Student's two-tailed t test.

Measurement of SOD activity, malondialdehyde MDA content and relative electric conductivity of leaves

Measurement of SOD activity, MDA content and relative electric conductivity was conducted at 0, 2, 6, 12, 18 d, respectively, after salt treatment. Total SOD activity was assayed according to the method of Jiang et al. (Jiang and Zhang, 2002). MDA was determined by a color reaction with thiobarbituric acid (Heath and Packer, 1968). To measure ion leakage ratio as relative electric conductivity parameter, 0.1 g same positional leaves were removed from different plants, rinsed briefly with deionized water and immediately placed into a tube with 10 mL of deionized water. Conductivity (I_1) was measured using an electroconductivity meter (model 1054, VWR Scientific, Phoenix) after the tubes were placed at 22℃ overnight. Then, the samples were heated at 100℃ for 30 min and conductivity (I_2) was measured again. Ion leakage ratio was expressed as (I_1/I_2) ×100%. For all the above measurements, three replicates per line were used. Statistical differences were determined using Student's two-tailed t test.

RESULTS

Molecular characterization of transgenic plants

After floral dip transformation, 68 individual kanamycin

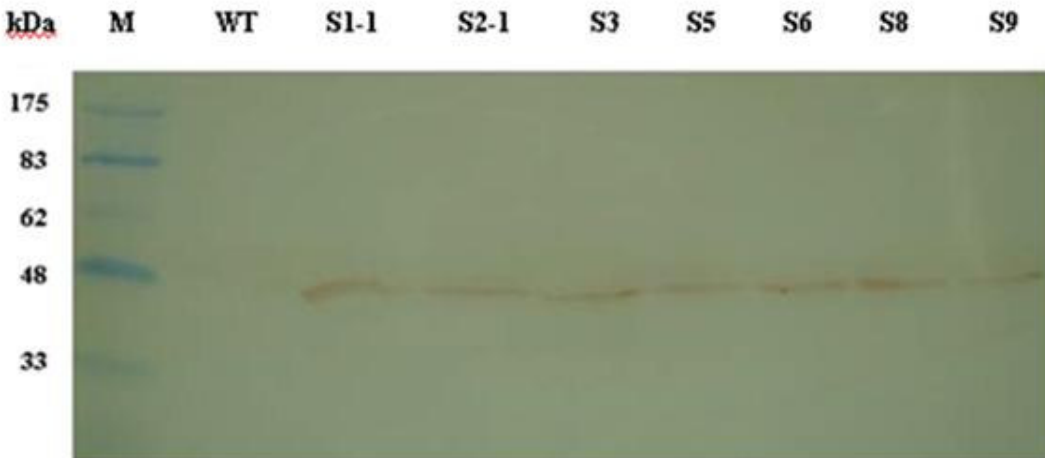

Figure 1. Western blot analysis of the *ZmPti1* gene expression in wild type *Arabidopsis* plants and seven *ZmPti1* homozygous transgenic lines. M, protein molecular weight marker; WT, wild type *Arabidopsis*; seven transgenic lines: S1-1, S2-1, S3, S5, S6, S8, S9.

resistant plants were obtained from T_0 seeds. The kanamycin-resistant T_1 plants were transferred into pots. An initial PCR from T_1 plant DNA templates confirmed that most kanamycin-resistant plants possess the transformed *ZmPti1* gene (data not shown). Only those PCR positive T_1 plants were allowed to set T_2 seeds. From T_2 seeds, plants were selected on kanamycin medium and grown in pots for T_3 seeds. Populations of T_3 seeds were established and were tested as homozygous transformants. The chosen homozygous transformants were used for immunoblot analysis for further confirmation. As shown in Figure 1, all the PCR positive plants had a specific HA-reactive band with the molecular mass of about 44 kD (Figure 1). This not only showed that the PCR positive plants were transgenic plants but also showed that ZmPti1-*dHA-His6* fusion protein had been expressed in transgenic plants.

ZmPti1 transgenic *Arabidopsis* plants increase salt tolerance

It had been reported that *ZmPti1* can be induced by salt stress. So it is interesting to know whether salt stress can cause any phenotypic changes in the *ZmPti1* transgenic plants. Two transgenic lines (S1-1, S2-1) and WT plants were treated with NaCl on MS agar plates (Figure 2) and in pots (Figure 3), the phenotypic changes were characterized.

One week after salt treatment on MS agar plates, the WT plants wilted to death, while S1-1 and S2-1 kept alive and their leaves were still green (Figure 2). This result primarily showed that *ZmPti1*-transgenic *Arabidopsis* increased salt tolerance.

To obtain further evidence that over-expression of *ZmPti1* conferred resistance to salt stress, the same two transgenic lines (S1-1 and S2-1) were selected for further salt stress analysis as described in materials and methods (Figure 3). After two weeks of salt treatment, the plants were photographed (Figure 3B). After salt treatment, 75% S1-1 plants and 72% S2-1 plants could flower and set seeds, while the rate of WT plants was 36%. These results further indicated that over-expression of *ZmPti1* in *Arabidopsis* increased salt tolerance.

Effect of constitutive *ZmPti1* expression on the fresh weight, dry weight and seeds weight under salt stress

The same two transgenic lines (S1-1 and S2-1) and WT plants were cultivated under salt stress or normal condition. After treatment, the above plants were harvested for seedling FW and DW measurement. Under normal condition, seedling FW and DW of transgenic plants were lower than that of WT plants (Figure 4). Under salt condition, seedling FW of S1-1, S2-1 plants was 0.20 and 0.21 g per plant, respectively, significantly higher than that of WT plants which was 0.14 g per plant (Figure 4A). Similarly, seedling DW of S1-1, S2-1 plants was 30 and 28.8 mg per plant, respectively, significantly higher than that of WT plants which was 11.3 mg per plant (Figure 4B). This result showed that over-expression of *ZmPti1* enhanced fresh and dry weight under salt treatment in *Arabidopsis.*

Salt tolerance is a very important limiting factor in terms of economic yields and seed weight is often considered as the representative factor of economic yields. Under normal condition, seed weight of S1-1, S2-1 plants was 3.75 and 3.63 g/100 plants, respectively, slightly lower than that of WT plants which was 4.06 g/100 plants (Figure 5). Under the treatment of 300 mM NaCl, seed weight of S1-1, S2-1 plants was 0.35 and 0.29 g/100 plants, respectively, significantly higher than that of WT

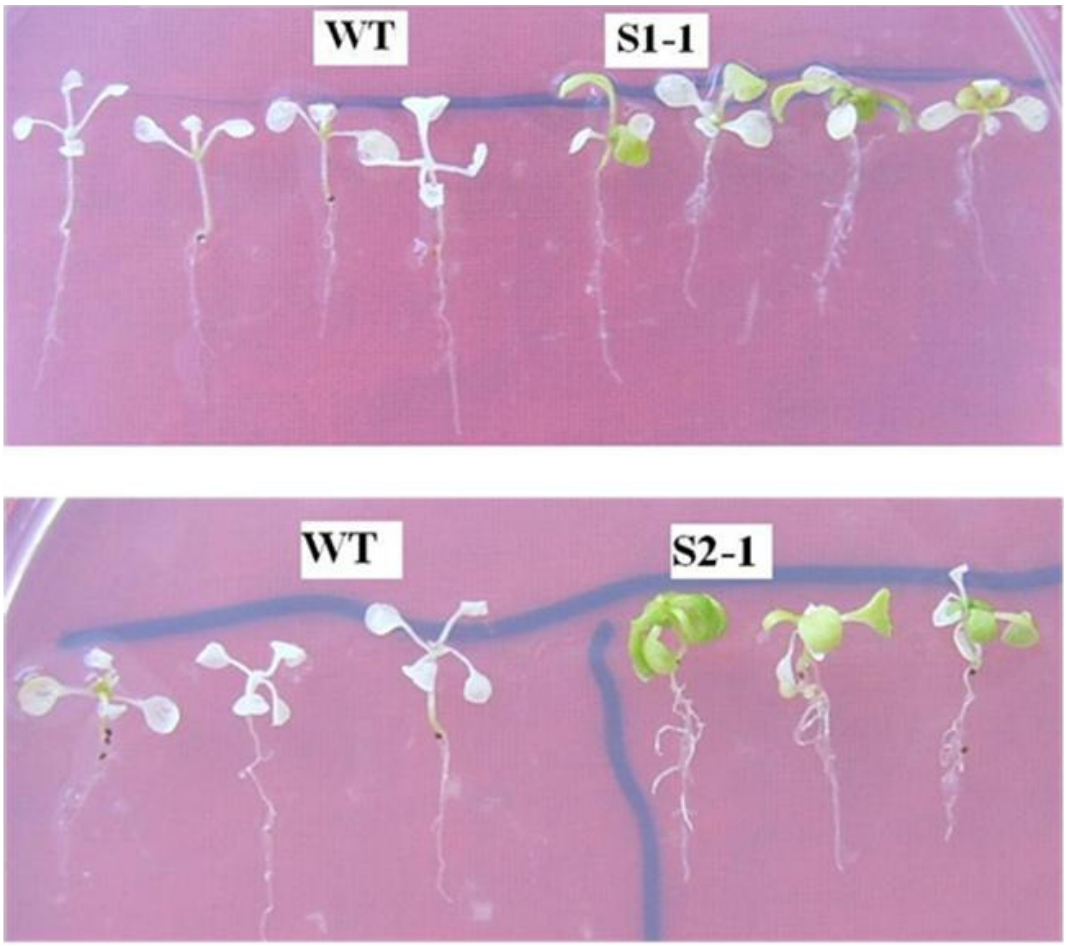

Figure 2. Comparison of wild type (WT) *Arabidopsis* plants and two *ZmPti1* transgenic lines (S1-1, S2-1) growing on MS agar plates containing 200 mM NaCl for one week.

plants which was 0.17 g/100 plants; seed weight of S1-1, S2-1 plants increased 106 and 71% respectively (Figure 5).

SOD activity, MDA content and ion leakage ratio in ZmPti1 transgenic *Arabidopsis* plants under salt stress

Salt stress can lead to many physiological responses in plants, such as activities of antioxidative enzymes, lipid peroxidation and ion leakage ratio (Mittova et al., 2004; Wang et al., 2009). In this experiment, SOD activity, MDA content and ion leakage ratio in transgenic and WT plants under salt treatment were analyzed. Under normal condition, SOD activity of transgenic plants was significantly higher than that of WT plants (Fig. 6A). Then, they both increased till the third day of salt treatment, but the improvement of SOD activity of transgenic lines was higher than that of WT plants. After that, the SOD activity of both transgenic and WT plants decreased rapidly to low levels, while the SOD activity of transgenic plants was still significantly higher than that of WT plants before 12 days treatment. This showed that transgenic plants could keep the SOD activity to higher levels for a long time than WT plants.

MDA is the product of lipid peroxidation, its content in plants reflects the degree of cell injury (Pérez-Tornero et al., 2009). As shown in Figure 6B, under normal condition, MDA content in both transgenic and WT plants was kept at a lower level and there was no obvious difference among them. After three days of salt treatment, MDA content in WT plants increased rapidly. However, MDA content in transgenic plants increased gradually in twelve days of salt treatment, after that, it then increased rapidly. Moreover, under salt condition, MDA content in transgenic plants was significantly lower than that in WT plants from 6 to 18 days after salt treatment. This result indicated that over-expression of *ZmPti1* gene could decrease the injury of plants under salt stress.

Ion leakage ratio also reflects the degree of plant cell injury under stress treatment. There was no obvious difference among the ion leakage ratio of the tested plants under normal condition (Figure 6C). Between 3 - 6 days

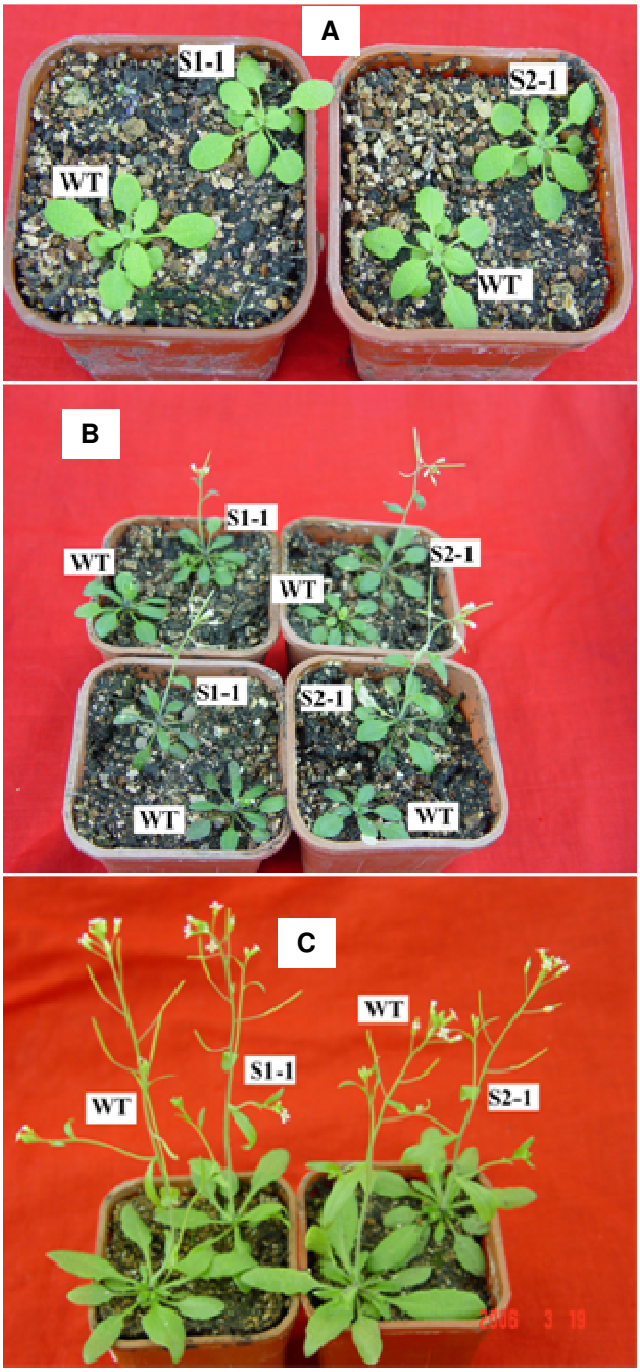

Figure 3. Phenotype of *ZmPti1* transgenic plants under salt stress. (A) Growth status of wild type (WT) *Arabidopsis* plants and two transgenic lines (S1-1, S2-1) before salt treatment; (B) Comparison of the growth of WT plants and two transgenic lines after two weeks of salt treatment; (C) Growth of two transgenic lines and WT plants under normal condition.

of salt treatment, ion leakage ratio of transgenic plants was significantly lower than that of WT plants (Figure 6C). Although after 12 days of salt treatment, ion leakage ratio of transgenic plants was close to that of WT plants, but still lower.

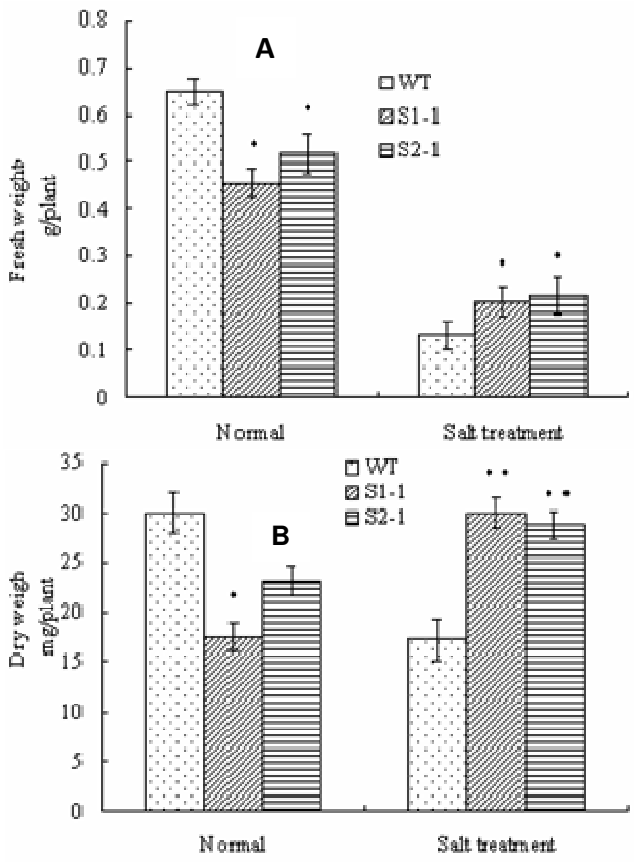

Figure 4. Fresh weight (A) and dry weight (B) of wild type (WT) *Arabidopsis* plants and two *ZmPti1* transgenic lines (S1-1, S2-1) under normal or salt treatment. Error bars indicate ±SE (n = 3). * and **, Significantly different from the WT at $P < 0.05$ and < 0.01, respectively, by Student's *t* test.

DISCUSSION

Previous work focused on the function of Pti1 in disease defense signal pathway (Zhou et al., 1995; Tian et al., 2004; Sessa et al., 1998). And our work suggested that ZmPti1 was also induced by SA which had been considered to be involved in plant disease resistance (Delaney et al., 1994; Durner et al., 1997). So, we first analyzed whether over-expression of *ZmPti1* gene could enhance the pathogen tolerance. However, when syringe-infiltrated with *Pseudomonas syringae* pv. tomato DC3000, the transgenic plants did not exhibit higher tolerance than WT plants (data not shown). This indicated that ZmPti1 might not play important roles in disease resistance pathway in *Arabidopsis*.

Experiments were performed for the evaluation of salt tolerance and differences between the transgenic lines and WT plants. On MS agar plates supplemented with 200 mM NaCl, WT plants displayed growth inhibition and all died after 1 week of salt treatment. Meanwhile, leaves of transgenic plants still kept green at this time, although they also displayed growth inhibition to some degree

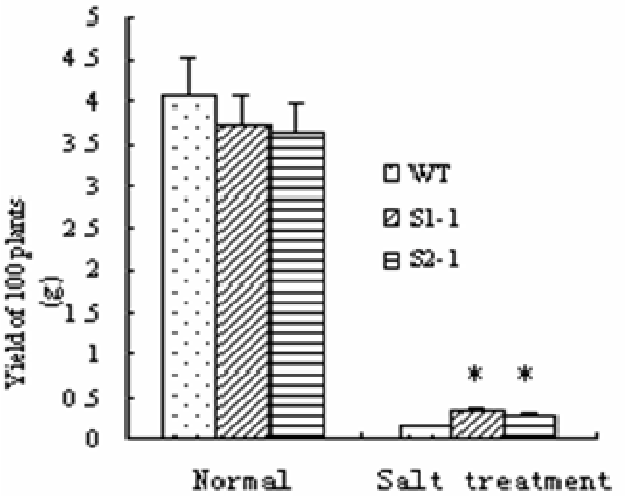

Figure 5. Seed weight of wild type (WT) *Arabidopsis* plants and two *ZmPti1* transgenic lines (S1-1, S2-1) under normal or salt treatment. Error bars indicate ±SE (n = 3). * and **, Significantly different from the WT at $P < 0.05$, and < 0.01, respectively, by Student's *t* test.

(Figure 2). Biomass and economic yields are useful traits to use for stress tolerance evaluation. In this study, when treated with NaCl solution, 75% of transgenic line S1-1 completed their life cycle and set seeds, the other transgenic lines showed similar phenotype. But only 36% of WT plants could complete life cycle and set seeds. Furthermore, seedling fresh weight and dry weight, seed weight of transgenic plants were also significantly higher than those of WT plants. These showed that *ZmPti1* might play an important role in salt resistance pathway.

Studies had shown that like other abiotic stresses, salinity also induces oxidative stress in plants (Holmberg and Bulow, 1998; Hasegawa and Bressan, 2000; Mittova et al., 2004). This oxidative stress can lead to lipid peroxidation and membrane permeabilization and as a result, ion leakage ratio and MDA content in the living cells increases (Scandalios, 1993; Pérez-Tornero et al., 2009). SOD forms the first step in the removal of the reactive oxygen intermediates (ROIs); it rapidly removes $O^{\bullet-}_2$ and hence decreases the risk of the formation of $^{\bullet}OH$ from $O^{\bullet-}_2$. Its role in providing protection to plants against oxidative damage has been well established. A few studies, nevertheless, showed a positive correlation between SOD activity level and salt tolerance (Rout and Shaw, 2001). Moreover, transgenic plants that over-express ROS-scavenging enzymes, for example, SOD, POD, CAT, GSH led to improved salt tolerance because of enhanced ROS scavenging and the prevention of membrane damage (Chinnusamy et al., 2005; Wang et al., 2009). In this study, when treated with salt, SOD activity in transgenic plants was higher than that in WT plants, however, ion leakage and MDA content in transgenic plants was lower than that in WT plants. This indicated that *ZmPti1* might enhance salt tolerance by increasing

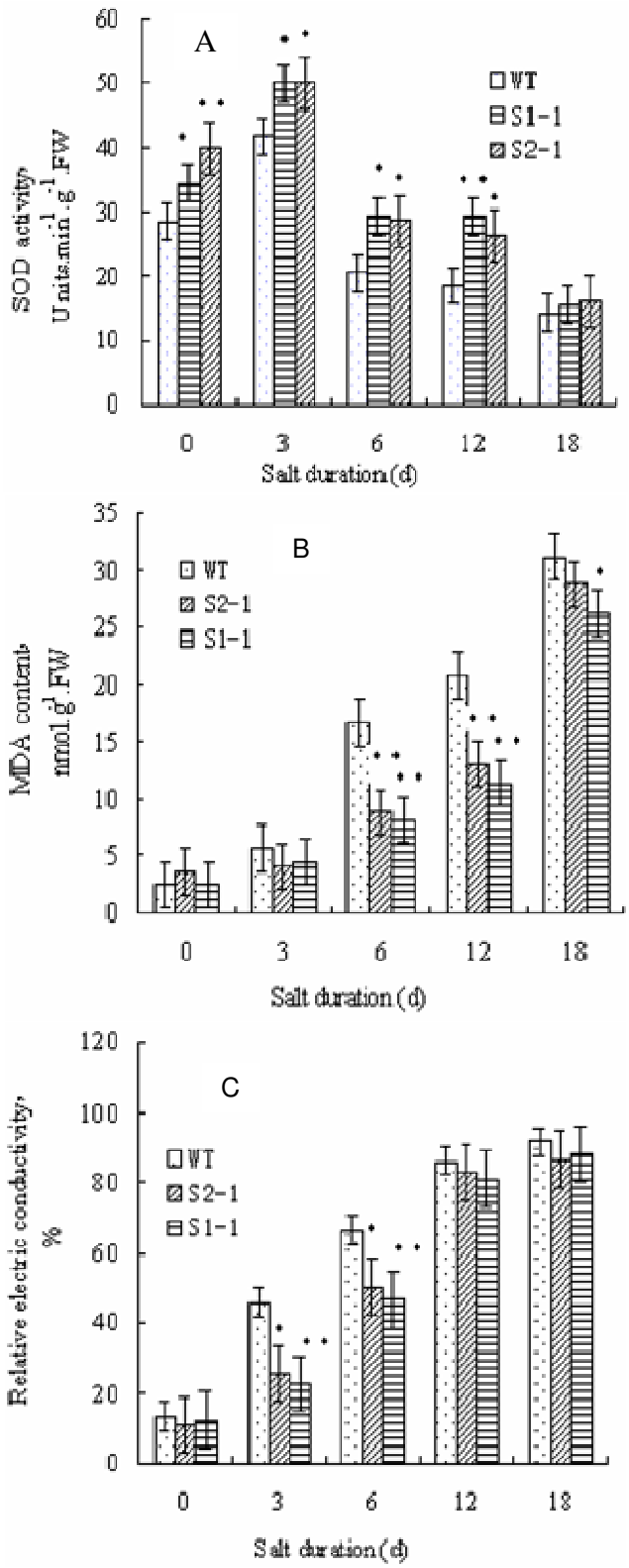

Figure 6. Changes of some biochemical parameters in leaves of WT *Arabidopsis* plants and two *ZmPti1* transgenic lines (S1-1, S2-1) under normal or salt treatment. (A) SOD activity, (B) MDA content, (C) ion leakage ratio. Error bars indicate ±SE (n = 3). * and **, Significantly different from the WT at $P < 0.05$, and < 0.01, respectively, by Student's *t* test.

SOD activity and reducing the injury of cells under salt stress. Also, we can conclude that *ZmPti1* might act as an upstream regulating element of ROS-scavenging enzymes in higher plants.

ACKNOWLEDGEMENTS

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